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hrp Genes of Phytopathogenic Bacteria

U. Bonas

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1 Introduction

In nature plants are resistant to the majority of pathogens, and many bacteria live in close contact with the plant without causing any harm (see chapter by Beattie and Lindow in this volume). Among the 1600 different species known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. Only a few of these species are gram-positive, e.g., *Clavibacter* ssp. and *Streptomyces* ssp. In this review I focus on subspecies of the gram-negative genera *Erwinia*, *Pseudomonas*, and *Xanthomonas*, which comprise the major bacterial plant pathogens.

To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living plant tissue. In nature, bacteria enter the plant through natural openings (stomata, hydathodes) or

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wounds. The bacterial armory contains a number of weapons that contribute to pathogenicity. Obvious examples include degradative extracellular enzymes such as pectinases, cellulases, and proteases. When the corresponding genes are mutated, bacterial ability to invade plant tissues is more or less affected depending on the pathogen, i.e., these functions contribute to and modulate development and severity of infection to different extents (see chapters by Dow and Daniels, and Collmer and Bauer in this volume).

In addition, phytopathogenic bacteria possess a large number of genes needed for basic pathogenicity. These genes have been operationally defined as *hrp* (hypersensitive reaction and pathogenicity; LINDGREN et al. 1986) based on their mutant phenotype. *hrp* genes are not only essential for pathogenicity on a plant, i.e., the ability to cause disease in a compatible interaction, but also for the incompatible interaction with resistant host varieties or with plants that are not normally a host for the particular pathogen (so called non-host). The incompatible interaction is often associated with the induction of a hypersensitive reaction (HR) in the plant. In contrast to the use of the term hypersensitivity in the animal field, in plants the HR is a rapid defense response involving localized plant cell death, production of phenolics and antimicrobial agents, e.g., phytoalexins, at the site of infection (KLEMENT 1982; LINDSAY et al. 1993). The HR results in prevention of pathogen multiplication and spread and thus in prevention of disease development. Under natural infection conditions the HR is microscopically small and can be induced by just one bacterial cell. Only when bacteria are introduced into plant tissue at high cell densities in the laboratory (about 10^7 colony forming units or more/ml) is the HR macroscopically visible as confluent necrosis and can be clearly distinguished from typical disease symptoms. It is important to note that saprophytic or nonpathogenic bacteria such as *Escherichia coli* or *Pseudomonas fluorescens* do not induce the HR and are unable to multiply in plant tissue.

2 Isolation of *hrp* Genes and General Features

hrp genes have been isolated from all major gram-negative plant pathogenic bacteria except Agrobacterium. There are excellent reviews that describe the early work or focus more on one particular pathogen (WILLIS et al. 1991; BOUCHER et al. 1992). The majority of *hrp* genes have been identified by complementation of loss-of-function mutants. Mutants obtained by random chemical (e.g., N-methyl-*N'*-nitro-*N*-nitrosoguanidine) or transposon mutagenesis of a pathogenic wild-type strain were inoculated into the host plant and screened for loss of both the ability to cause disease in susceptible plants and to induce the HR in resistant host or non-host plants (often tobacco). The second criterion for the isolation of genes specific for the plant interaction was to ensure that the mutants would still grow in minimal medium. This way mutants affected in genes for basic housekeeping functions were eliminated. A third characteristic of all *hrp* mutants is that they are unable to grow in the plant.

The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. LINDGREN and coworkers (1986) isolated Tn*5*-induced mutants of *P.s.* pv. *phaseolicola* that had lost both the ability to induce halo-blight disease on bean and the HR in tobacco. Complementation with cosmid clones from a genomic library of the wild-type strain resulted in isolation of a cluster of *hrp* genes localized in a 20 kb DNA region. This was the first indication that both the ability to cause disease and to induce the HR are mediated by common steps in a "pathway".

Since then *hrp* gene clusters have been cloned from a number of different bacteria. Examples include *Pseudomonas solanacearum* (BOUCHER et al. 1987; Fig. 1B), the *Xanthomonas campestris* pathovars *campestris* and *vitians* (ARLAT et al. 1991), *translucens* (WANEY et al. 1991), and *vesicatoria* (BONAS et al. 1991; Fig. 1A), *Erwinia amylovora* (STEINBERGER and BEER 1988; BARNY et al. 1990; WALTERS et al. 1990; BAUER and BEER 1991), and several other pathovars of *P. syringae* (e.g., HUANG et al. 1988; LINDGREN et al. 1988; Fig. 1C). In addition, genes with DNA homology, and in some cases functional homology, have been isolated from other species, e.g., the so-called *wts* genes from *E. stewartii* (COPLIN et al. 1992; LABY and BEER 1992), and a region containing pathogenicity genes from *X.c.* pv. *glycines* that complement *hrp* mutants of *X.c.* pv. *vesicatoria* (HWANG et al. 1992; Bonas, unpublished results). Interestingly, nonpathogenic xanthomonads that were originally isolated from diseased plants as opportunists together with pathogenic bacteria do not contain *hrp*-related DNA sequences (STALL and MINSAVAGE 1990; BONAS et al. 1991). In *Agrobacterium tumefaciens* or in strains of *Rhizobium* ssp. there seem to be no *hrp* gene equivalents present (BONAS et al. 1991; LABY and BEER 1992). This conclusion is based on DNA hybridization experiments and, of course, does not exclude the presence of genes with functional homology to *hrp* genes in these species.

In all of the cases mentioned above, the *hrp* genes are organized in clusters of 22–40 kb, and I will restrict most of this chapter to these large *hrp* clusters. In addition, several smaller *hrp* loci have been described that are not linked to the large cluster present in the same bacterium. These include a region in *P. solanacearum* (HUANG et al. 1990), the *hrpX* locus that is conserved in *X. campestris* pathovars *campestris* (KAMOUN and KADO 1990; KAMOUN et al. 1992) and *oryzae* (KAMIJAH et al. 1993), and the *hrpM* locus in *P.s.* pv. *syringae* (NIEPOLD et al. 1985; MUKHOPADHYAY et al. 1988). *hrpM* is functionally conserved in pathovar *phaseolicola* (FELLAY et al. 1991). Besides being nonpathogenic and unable to induce the HR in tobacco, *P. syringae hrpM* mutuants are also affected in mucus production. The *hrpM* locus encodes two putative proteins which are similar and have been shown to be functionally homologous to the products of the *E. coli mdoGH* operon (LOUBENS et al. 1993). The *mdoGH* genes are required for periplasmic membrane-derived oligosaccharide synthesis in *E. coli*. The *hrpQ* and *hrpT* genes from *P.s.* pv. *phaseolicola* (MILLER et al. 1993) will be discussed later in this chapter.

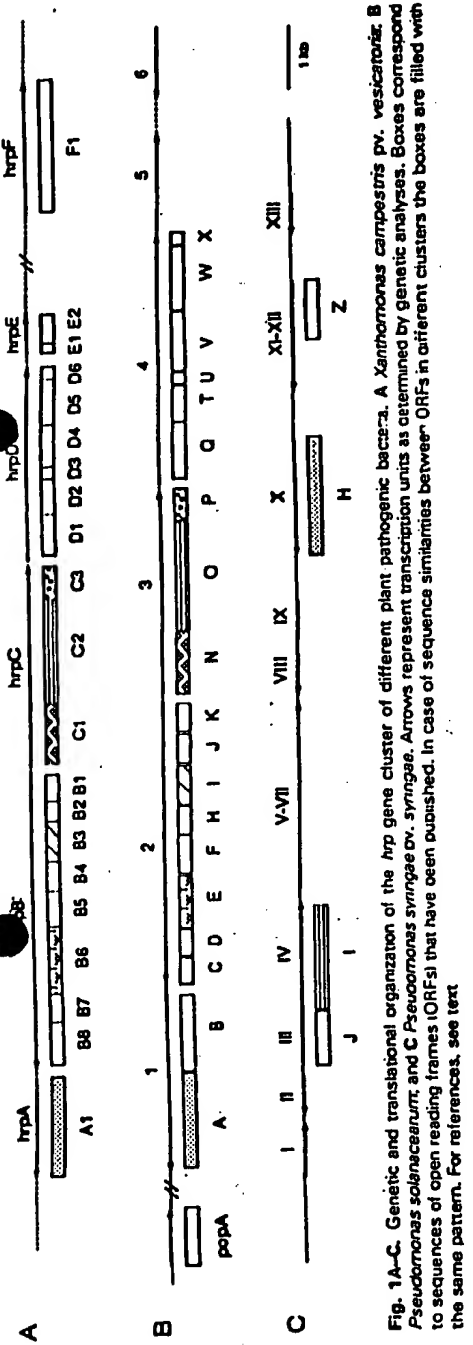

Fig. 1A–C. Genetic and translational organization of the *hrp* gene cluster of different plant pathogenic bacteria. A *Xanthomonas campestris* pv. *vesicatoria*; B *Pseudomonas solanacearum*; and C *Pseudomonas syringae* pv. *syringae*. Arrows represent transcription units as determined by genetic analyses. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern. For references, see text

3 Structural Organization and Relatedness of *hrp* Clusters

Genetic studies using transposon-induced insertion mutants in the respective bacterial wild-type strains revealed that the *hrp* clusters contain at least six to eight complementation groups (Fig. 1). Some *hrp* gene clusters have clearly been shown to be localized in the chromosome, e.g., in *P.s.* pv. *phaseolicola* (Rahme et al. 1991) and in *X.c.* pv. *vesicatoria* (Bonas et al. 1991), whereas in *P. solanacearum*, the *hrp* cluster is on a megaplasmid (Boucher et al. 1987).

Striking similarities have recently been found between the *hrp* genes of pathogens belonging to different genera. The first indication of homologies came from Southern hybridization studies. DNA homology was observed among different strains of the same pathovar, as well as between pathovars or strains within a species, and in some cases also between species. However, the degree of conservation varies. DNA homology is high within pathovars of a given subspecies, e.g., in *P. syringae* (Lindgren et al. 1988; Huang et al. 1991) and in *X. campestris* (Bonas et al. 1991). The latter studies were recently extended by PCR using primers based on *hrp* sequences from *X.c.* pv. *vesicatoria* (Leite et al. 1994). Furthermore, at least some regions of the *hrp* clusters appear to be conserved on the DNA level between *P. solanacearum* and pathovars of *X. campestris*, *P. syringae*, and also to *E. amylovora* (Boucher et al. 1987; Arlat et al. 1991; Gough et al. 1992; Laby and Beer 1992). In addition, cross-complementation within a subspecies indicated a high degree of functional conservation of *hrp* genes (e.g., Lindgren et al. 1988; Arlat et al. 1991; Bonas et al. 1991; Laby and Beer 1992). Due to sequence data it is now becoming more and more apparent that several *hrp* genes are conserved in all major gram-negative plant pathogenic bacteria (see below). Whether there are *hrp* genes that are clearly pathovar-specific can only be answered when complete sequence information becomes available for several *hrp* clusters.

4 Function of *hrp* Genes in *Xanthomonas campestris* pv. *vesicatoria* and Other Plant Pathogenic Bacteria

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. One of the first genes sequenced was a regulatory gene, *hrpS*, from *P.s.* pv. *phaseolicola* (Grimm and Panopoulos 1989). This gene as well as *hrpB*, a regulatory gene from *P. solanacearum* (Genin et al. 1992), will be discussed below in the context of gene regulation.

Since *hrp* genes are environmentally regulated (see below), it was believed for a while that they would be encoding "alternative" proteins required for adaptation of the bacterium to the plant as the preferred environment. The recently discovered sequence similarities between several putative Hrp proteins and known proteins from other bacteria, however, led to a very different hypothesis, namely, involvement of Hrp proteins in protein secretion. We have

sequenced the entire *hrp* cluster of *X.c.* pv. *vesicatoria*. Since most *hrp* sequences from this and other bacteria are not yet published, I will summarize our results and refer to the other phytopathogenic bacteria as I go along. Based on genetic analyses and the open reading frames (ORFs) with a high coding probability we predict 21 *hrp* genes in the 25 kb *hrp* cluster of *X.c.* pv. *vesicatoria*. Their transcriptional organization is depicted in Fig.1A. The loci *hrpA* and *hrpB* are transcribed from right to left; the other four loci are transcribed from left to right (SCHULTE and BONAS 1992a). According to the locus (*hrpA–hrpF*) we have numbered the ORFs consecutively. The *hrpA* locus appears to contain just one *hrp* gene, *hrpA1*. The *hrpB* operon contains eight ORFs, called *hrpB1–hrpB8*, etc. A region of about 4 kb between *hrpE* and *hrpF* does not seem to be involved in the interaction with the plant because insertions in this region do not lead to a change in phenotype (BONAS et al. 1991).

What are the characteristics of the Hrp proteins? It should be noted that, except for three proteins, expression of the other 18 has yet to be demonstrated in *X.c.* pv. *vesicatoria*. A number of putative Hrp proteins are most likely associated with or localized in the bacterial membrane. For example, the HrpC2 protein sequence contains eight transmembrane domains but lacks a signal sequence, suggesting an inner membrane localization (FENSELAU et al. 1992). Both HrpA1 and HrpB3 contain an NH_2-terminal signal sequence and one (HrpA1) or two (HrpB3) transmembrane domains, suggesting that a part of these proteins might be targeted to the outer membrane. The signal sequence of HrpB3 resembles signal peptidase II sequences which are typical of lipoproteins (FENSELAU et al. 1992). Experiments using radioactively labeled palmitate are underway to test whether HrpB3 is a lipoprotein. Recently, both HrpB3 and HrpA1 were shown to be localized in the *X.c.* pv. *vesicatoria* membrane fraction using polyclonal antibodies (S. Fenselau, C. Marie, and U. Bonas, manuscript in preparation). The HrpB6 protein is a putative ATPase with highly conserved nucleotide and magnesium binding domains. It is more similar to protein traffic ATPases than to proton pump ATPases, and the lack of membrane spanning domains suggests a cytoplasmic location (FENSELAU et al. 1992).

Searches of the database revealed sequence relatedness of more than half of the *X.c.* pv. *vesicatoria* Hrp proteins with putative proteins in other bacteria, including different plant pathogens. High DNA sequence identity (more than 90%) was found to a 2.7 kb fragment carrying pathogenicity genes from *X.c.* pv. *glycines* (HWANG et al. 1992). The authors predicted two ORFs, whereas in *X.c.* pv. *vesicatoria*, this region contains three ORFs corresponding to the *hrpC3*, *hrpD1* and *hrpD2* genes. Complementation studies indicated that part of the *hrp* region is colinear in the two pathovars of *Xanthomonas* (unpublished).

The deduced amino acid sequences of *hrp* genes published from *P. solanacearum* (GOUGH et al. 1992, 1993; GENIN et al. 1992) show significant similarity to *X.c.* pv. *vesicatoria* proteins (Table 1; Fig.1). One exception is the *hrpB* regulatory gene from *P. solanacearum* which is not present in the 25 kb *hrp* region or in the flanking region of the *X.c.* pv. *vesicatoria hrp* cluster as determined by DNA sequence analysis and hybridization studies (T. Horns and U. Bonas, unpublished). Furthermore, several of the proteins mentioned are conserved in other species (Fig.1), however, the degree of sequence similarity varies greatly (Table 1). The HrpA1 protein from *X.c.* pv. *vesicatoria* is 48% and 29% identical to proteins from *P. solanacearum* (HrpA; GOUGH et al. 1992) and *P.s.* pv. *syringae* (HrpH; HUANG et al. 1992), respectively. HrpC2 from *X.c.* pv. *vesicatoria* is even more conserved, being 66% idential to the corresponding HrpO protein of *P. solanacearum* (GOUGH et al. 1993), whereas the *hrpI* genes from *E. amylovora* (WEI and BEER 1994) and from *P.s.* pv. *syringae* (HUANG et al. 1993) both show 62% similarity to *hrpC2* from *X.c.* pv. *vesicatoria*. *P.s.* pv. *syringae* also contains a *hrpB3* related gene, called *hrpY*, and a *hrpD2* related gene, *hrpW* (H.-C. Huang, personal communication). Thus, the high degree of DNA sequence conservation that was reported earlier (see above) is also seen on the protein level. It appears that *hrp* genes in *X.c.* pv. *vesicatoria* are more closely related to *P. solanacearum* than to *P. syringae* and to *Erwinia*. As more and more homologous *hrp* genes are found in other bacteria nomenclature might become confusing. However, as long as the genes have not been shown to be functionally homologous, we will continue to use these names.

Besides genes that are conserved among the major phytopathogenic bacteria some genes are absent in the *hrp* region of more distantly related species. For example, there are no known homologs of the harpin genes *hrpN* (WEI et al. 1992a), and *hrpZ* (HE et al. 1993) (see below), and of *hrpJ* from *P.s.* pv. *syringae* (HUANG et al. 1993) in the *X.c.* pv. *vesicatoria hrp* cluster (unpublished; see Fig. 1).

Similarities of 50%–60% were found recently between HrpA1 and HrpB3 from *X.c.* pv. *vesicatoria* and two putative NoI proteins of *Rhizobium fredii* that are encoded by a cultivar specificity region. NolT and NolW mutants have a wider host range in nodulation of soybean (MEINHARDT et al. 1993). In addition, the authors mention that release of proteins is affected.

Last but not least, Table 1 summarizes the significant sequence similarities which have been found to proteins from animal bacterial pathogens. A number of putative Hrp proteins are related to proteins in animal pathogens such as *Salmonella*, *Shigella*, and *Yersinia* ssp. Since the first similarities found were to the Ysc, Vir, and Lcr proteins from *Yersinia* ssp, this group of organisms became a "role model" for plant pathologists (FENSELAU et al. 1992; GOUGH et al. 1992; HUANG et al. 1992). In *Yersinia*, these proteins are essential for the secretion of virulence factors, called Yops (Yersinia outer protein; MICHIELS et al. 1990, 1991). Since they are described in detail in the chapter by G.R. Cornelis, I will mention only a few important features. The Yops are hydrophilic proteins that lack a typical NH_2-terminal signal peptide, and are secreted by using an entirely different pathway from that previously described for protein secretion. The genes involved in secretion are clustered on a 70 kb virulence plasmid. In case of a mutation, e.g., in *YscJ*, the Yops accumulate in the cytoplasm (MICHIELS et al. 1991). Although their direct role in transport has yet to be demonstrated, it is believed that the Ysc and Lcr proteins mentioned in Table 1 are parts of a special transport apparatus for Yop secretion. Similarly, *Shigella flexneri* secretes virulence factors, called Ipa (invasion plasmid antigens), that are distinct from Yops but share the general

Table 1. Sequence similarities of *Xanthomonas campestris* pv. *vesicatoria* Hrp proteins

Xanthomonas campestris pv. *vesicatoria*	HrpA1[1]	HrpB6[1]	HrpB3[1]	HrpC1[1]	HrpC2[1]	HrpC3[1]	HrpD1[1]	HrpD2[1]
Pseudomonas solanacearum	HrpA[3] (66%)	HrpE[4]	Hrpl[5] (70%)	HrpN[6] (74%)	HrpO[3] (81%)	HpaP[5] (54%)	HrpQ[4]	HrpT[4]
Pseudomonas syringae pv. *syringae*	HrpH[6] (52%)				Hrpl[7] (62%)			
Yersinia enterocolitica	YscC[8] (55%)		YscJ[8] (56%)					
Yersinia pestis	YscC[9] (55%)				LcrD[10] (70%)		LsaA[11] (52%)	LsaB[11] (72%)
Yersinia pseudotuberculosis		YscN[12] (73%)	LcrKa[13] (56%)					
Shigella flexneri	MxiD[14] (50%)	Spa47[15] (65%)	MxiJ[16] (52%)	Spa40[17] (55%)	MxiA[18] (65%)			Spa24[19] (67%)
Salmonella typhimurium	InvG[19] (52%)	SpaL[20] (70%)		SpaS[20] (56%)	InvA[22] (67%)			SpaP[20] (64%)
		FliI[23] (65%)						
Bacillus subtilis		FlaA-ORF4[24] (68%)		FlhB[25] (62%)	FlhA[25] (63%)			FliP[26] (68%)
Escherichia coli		β-F1[27] (53%)						FliP[28] (65%)
Erwinia carotovora							MopB[29] (49%)	MopC[29] (65%)
Erwinia amylovora					Hrpl[30] (62%)			
Rhizobium fredii	NolW[31] (51%)		NolT[27] (61%)					
Caulobacter crescentus					FlbF[32] (55%)			

Similarities between deduced amino acid sequences of Hrp proteins from *X.c.pv.vesicatoria* and other proteins include conservative amino acid exchanges. Number in parentheses indicates percent similarity.

Superscript numbers indicate references as follows:

1. Fenselau et al. 1992; 2.Bonas et al., unpublished; 3. Gough et al. 1992; 4. Genin et al. 1993, sequences unpublished; 5. Gough et al. 1993; 6. Huang et al. 1992; 7. Huang et al. 1993; 8. Michiels et al. 1991; 9. Haddix and Straley 1992; 10. Plano et al. 1991; 11. Fields et al. unpublished, accession # L22495; 12. Galyov, unpublished, accession # U00998; 13. Rimpilainen et al. 1992; 14. Allaoui et al. 1993; 15. Venkatesan et al. 1992; 16. Allaoui et al. 1992; 17. Sasakawa et al. 1993; 18. Andrews and Maurelli 1992; 19. Lodge et al., unpublished, accession # X75302; 20. Groisman and Ochman 1993; 21. Vogler et al. 1991; 22. Galán et al. 1992; 23. Albertini et al. 1991; 24. Carpenter et al., unpublished, accession # X741212; 25. Carpenter and Ordal 1993; 26. Bischoff et al. 1992; 27. Saraste et al. 1981; 28. Malakooti et al. unpublished, accession # L21994; 29. Mulholland et al. 1993; 30. Wei and Beer 1993; 31. Meinhardt et al. 1993; 32. Ramakrishanan et al. 1991; Sanders et al. 1992.

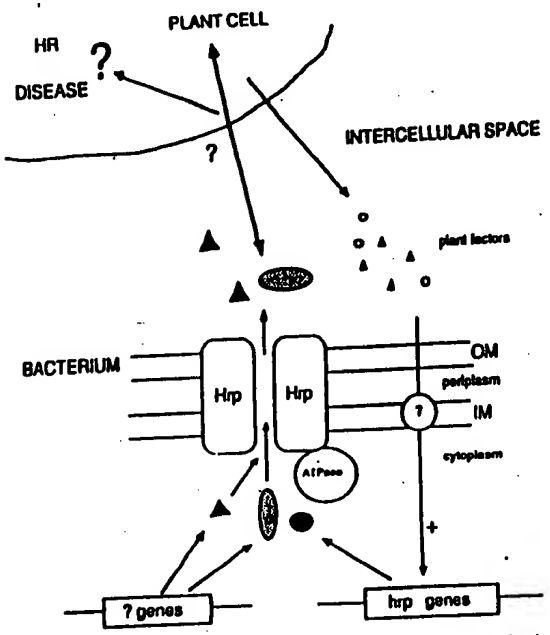

Fig. 2. Hypothetical model of cell signaling between gram-negative bacteria and plants indicating the proposed function of Hrp proteins as an apparatus for protein secretion. The model has been modified after FENSELAU et al. (1992). Hrp proteins may form a tunnel that enables the export of molecules such as virulence factors or avirluence factors leading to either a hypersensitive response (HR) or disease. These factors could be encoded by *hrp* genes or genes unlinked to the large cluster. Both types of genes have been found to encode elicitors of the HR (see text). The secretion of virulence proteins is hypothetical

features mentioned above (HALE 1991; and see chapter by PARSOT, this volume). Although *S. typhimurium* appears to possess a secretion system similar to that in *Shigella*, secreted invasion antigens have not yet been identified (GILLESMAN and OCHMAN 1993; see chapter by FINLAY). As unpublished reports indicate that more and more genes in the animal pathogens are conserved, the data shown in Table 1 will soon be out of date. Proteins from other bacteria, e.g., *E. coli*, *Bacillus*, *Caulobacter* and from the *mop* region in *E. carotovora* (MULHOLLAND et al. 1993), have also been found to be similar to Hrp proteins (Table 1). Most of these are important for the assembly of the flagella, motility, or chemotaxis, again pointing, in my opinion, to a specialized secretion system rather than an involvement of *hrp* genes in chemotaxis.

These observations led us and others to propose a *hrp*-dependent secretion system in plant pathogenic bacteria (FENSELAU et al. 1992; GOUGH et al. 1992; VAN GIJSEGEM et al. 1993). A model is shown in Fig. 2 and raises certain questions, e.g., if secretion occurs, what is being secreted by plant pathogenic bacteria? So far, a few proteins have been identified as elicitors of the HR but there is no evidence for secretion of virulence factors (see below).

5 *hrp*-dependent Secretion of Hypersensitive Response-Inducing Proteins

5.1 Harpin from *Erwinia amylovora*

An important feature of the isolated *hrp* clusters from both *E. amylovora* and *P.s.* pv. *syringae* is the ability of *E. coli* or *Pseudomonas fluorescens* transformants containing the cloned genes to induce the HR on tobacco (HUANG et al. 1988; BEER et al. 1991; see below). This has prompted to search for the HR-inducing activity within the respective gene clusters.

The first bacterial HR-inducing protein identified, designated harpin, is a cell envelope-associated protein encoded by the *hrpN* gene of *E. amylovora*, a pathogen of pear and apple (WEI et al. 1992a). This harpin$_{Ea}$ is a glycine-rich and heat-stable protein that induces the HR in the non-host, tobacco. The *hrpN* gene is localized within the respective *hrp* cluster and thus has a dual role in also being required for pathogenicity on the normal host plant. Its function in pathogenicity, however, is unknown. BEER et al. (1993) mentioned in a preliminary report that the *hrpN* gene seems to be conserved among *Erwinia* ssp. but that there is no DNA homology between *hrpN* and sequences in the other plant pathogenic bacteria. Although data described below suggest that the harpin$_{Ea}$ protein might be secreted via the Hrp secretory apparatus, there is no published information available that demonstrates this.

5.2 Harpin from *Pseudomonas syringae* pv. *syringae*

Using an elegant approach He and coworkers recently have identified harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P.s.* pv. *syringae* (HE et al. 1993; see Fig. 1C and chapter by Collmer and Bauer). Lysates of *E. coli* clones containing an expression library, made using the cloned *P.s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an NH_2-terminal deletion of harpin$_{Pss}$ with even higher activity than the full size protein. Whether or not processing occurs in natural infection is not clear. Interestingly, two short direct repeats in the COOH-terminus of harpin$_{Pss}$ are essential for elicitor activity. Although the two harpins harpin$_{Ea}$ and harpin$_{Pss}$ differ in their primary sequence, they have several features in common, e.g., a stretch of 22 amino acid that is similar in both proteins (HE et al. 1993). Harpin$_{Pss}$ is also glycine-rich and heat-stable. As with harpin$_{Ea}$ of *E. amylovora*, the function of harpin$_{Pss}$ in pathogenicity is unknown. Its product is secreted by *P.s.* pv. *syringae* in a HrpH-dependent way; HrpH is highly related to proteins involved in secretion in other plant and animal pathogens (HUANG et al. 1992; see Table 1).

5.3 *PopA* from *Pseudomonas solanacearum*

An HR-inducing protein has been identified and characterized from *P. solanacearum* culture supernatants, called Pop (Pseudomonas out protein; Arlat et al. 1994). PopA1 and two shorter derivatives, PopA2 and PopA3, induce the HR in tobacco and in certain, but not all, *Petunia* lines. Like the harpins, the Pop proteins are also heat-stable and glycine-rich, however, the sequence is entirely different. In contrast to the harpins, the *popA* gene is not a *hrp* gene but is located outside of the large *hrp* cluster. Interestingly, expression of *popA* is *hrpB*-dependent, i.e., the gene is part of the *hrp* regulon. Mutations in *popA* do not affect the HR on tobacco or pathogenicity on tomato suggesting that more than one HR-inducing factor is produced. Arlat et al. (1994) convincingly showed that secretion of PopA is dependent on other *hrp* genes, such as *hrpA*, *hrpN*, and *hrpO* (Fig. 1B). If a bacterial strain virulent towards *Petunia* is found it will be interesting to see if PopA acts as an avirulence protein in *Petunia* as has been suggested by the authors.

These exciting findings prove that certain Hrp proteins of *P.s.* pv. *syringae* and *P. solanacearum* play a role in transport of HR elicitors (Fig. 2). They also stimulate more questions. It needs to be shown that harpins and PopA are in fact secreted when the bacteria interact with the plant (the *hrp* genes were induced in vitro). Are harpins conserved among pathovars of *P. syringae*? How many elicitors f the n n-host HR in tobacco can be found? Is the mechanism of recognition in tobacc identical with the *Erwinia* and *P.s.* pv. *syringae* harpins and the *P. solanacearum* Pops?

6 Regulation of Expression of *hrp* Genes

Expressi n of *hrp* genes is controlled by environmental conditions and has been studied on the RNA level as well as using transcriptional fusions to reporter genes such as the *E. coli* genes encoding β-galactosidase or β-glucuronidase. In general, expression of *hrp* loci is not detectable when the bacteria are grown in complex culture media. However, after growth of the bacteria in the plant, *hrp* genes are expressed. Attempts to mimic the conditions that the different bacterial species encounter in the plant tissue resulted in the finding that growth in minimal media without any plant-derived factor was sufficient to induce *hrp* genes. This has led to the speculation that the bacteria have to experience some kind of starvation conditions for full expression of *hrp* genes. One of the first indications for *hrp* gene expression in vitro, and clearly a breakthrough, was a report on the *hrp*-dependent expression of an avirulence gene from the soybean pathogen *P.s.* pv. *glycinea* (Huynh et al. 1989).

Since the composition of minimal media differs depending on the bacterium studied, the most important findings will be summarized for representative pathogens. Parameters like carbon source, concentration of organic nitrogen and phosphate, osmolarity, and pH have been found to be important. High concentration of organic nitrogen generally appears to suppress *hrp* gene activation. Only two regulatory genes have been studied so far (see below). Interestingly, they both belong to different families of regulatory proteins.

6.1 *Pseudomonas syringae*

Expression of all seven *hrp* loci in the large cluster of *P.s.* pv. *phaseolicola* is suppressed in complex medium but induced in the plant. Induction occurs in the susceptible host plant as well as in the non-host, tobacco, suggesting that there is no plant species-specific molecule involved in control of host range (Rahme et al. 1992). Five complementation groups, *hrpAB*, *hrpC*, *hrpD*, hrpE and *hrpF*, can also be induced in M9 minimal medium containing sucrose as a carbon source, however, induction is affected by pH, osmolarity, and carbon source, and never reaches the levels obtained in the plant (Rahme et al. 1992). A similar observation was made earlier for the avirulence gene *avrB* in *P.s.* pv. *glycinea*. Induction occurred in a minimal medium containing fructose, mannitol, or sucrose. Expression of *avrB* is dependent on *hrp* genes homologous to *hrpRS* and *hrpL* from *P.s.* pv. *phaseolicola* and was suppressed by TCA cycle intermediates such as citrate and succinate (Huynh et al. 1989). *hrp* gene expression in *P.s.* pv. *syringae* occurs in the same medium as described by Huynh et al. (1989); (Huang et al. 1991; Xiao et al. 1992). The authors showed *hrp* gene induction in the non-host plant, tobacco, but no data for the host plant. The *P.s.* pv. *phaseolicola* loci *hrpL* and *hrpRS* are only expressed to a very low level in M9 minimal medium and are induced at least 1000-fold when the bacteria are inoculated into the plant. This led to the conclusion that, at least for expression of *hrpL* and *hrpRS*, specific plant factors might be necessary (Rahme et al. 1992).

6.2 Regulatory Genes *hrpRS* and *rpoN* of *Pseudomonas syringae* pv. *phaseolicola*

The results on environmental factors inducing or suppressing *hrp* gene expression suggested that specific regulatory genes are involved in the control of *hrp* promoter activities. At least two loci are involved in positive regulation of the other *hrp* loci of *P.s.* pv. *phaseolicola* *hrp* cluster (Fellay et al. 1991). While there is no information published for *hrpL*, *hrpRS* has been sequenced. It contains two genes whose predicted protein products are 60% identical to each other (Grimm and Panopoulos 1989; Miller et al. 1993). The HrpS protein is similar to members of the NtrC family of regulatory proteins in gram-negative bacteria. Most NtrC-like regulatory proteins are members of two-component systems, with a sensor protein that in turn activates a response element by phosphorylation of a site in the conserved NH_2-terminal domain (Albright et al. 1989). The putative sensor component operating in *hrp* gene regulation has not been identified. It is postulated that HrpS is the activating protein, however, direct biochemical data

have not been presented. The lack of a typical NH_2-terminal domain in HrpS could indicate that a different mechanism may be involved in HrpS activation. Apparently, *hrpS*-related sequences are also present in other bacteria, e.g., in *P.s.* pv. *syringae* (Heu and Hutcheson 1993) and in *Erwinia amylovora* (Beer et al. 1993). *E. stewartii* contains a transcriptional regulator, WtsA, with 52% identity to HrpS of *P.s.* pv. *phaseolicola*. The *hrpS* clone, however, was unable to functionally complement a *wtsA* mutant (Frederick et al. 1993).

The structure of the *hrpRS* locus and the finding of –24/–12 consensus sequences upstream of *hrpRS* indicated a possible role in transcriptional activation for transcription factor sigma 54, encoded by *rpoN* (Grimm and Panopoulos 1989). In a preliminary report, Felley et al. (1991) demonstrated that *hrp* gene expression in *P.s.* pv. *phaseolicola* is indeed dependent on *rpoN*. A *rpoN* mutant of *P.s.* pv. *phaseolicola* is a glutamine auxotroph and nonpathogenic. Whether *rpoN* is generally involved in regulation of *hrp* gene expression is not clear. In *X.c.* pv. *vesicatoria*, *rpoN* is clearly not involved in *hrp* gene expression and pathogenicity (T. Horns and U. Bonas, manuscript in preparation).

Recently, Miller et al. (1993) have reported the identification of two new loci, *hrpQ* and *hrpT*, from *P. s.* pv. *phaseolicola* that affect activation of *hrpRS* in *trans*. However, since *hrpRS* is strongly induced in plants while both *hrpQ* and *hrpT* are constitutively expressed, there must be more factors involved in *hrp* gene regulation. Strains carrying mutations in either *hrpQ* or *hrpT* are amino acid auxothrophs (methionine and tryptophan). *hrpQ* and *hrpT* are probably involved in methionine and tryptophan biosynthesis, respectively (Miller et al. 1993). As stated above, such mutants would normally have been eliminated from the *hrp* mutant analysis.

6.3 Conserved Sequence Boxes in *Pseudomonas syringae*

A conserved sequence, the so-called harp box (TG(A/C)AANC, Fellay et al. 1991), upstream of four *hrp* loci in *P. s.* pv. *phaseolicola*, was suggested to be involved in positive regulation of expression. Similar motifs were described for the promoter regions of several *P. syringae* avirulence genes, the expression of which is dependent on *hrpRS* and on *rpoN* (Huynh et al. 1989; Salmeron and Staskawicz 1993; Innes et al. 1993; Shen and Keen 1993). These studies led to a revised 'harp' box sequence (GGAACCNA). Its significance in protein binding has not been shown but *avrD* promoter constructs lacking the harp box are no longer inducible (Shen and Keen 1993). A harp box-related motif was also found upstream of transcription unit 3 in *P. solanacearum* (Gough et al. 1993).

There is no harp box sequence in *Xanthomonas hrp* gene promoters. Another sequence motif that occurs in the promoter region of *hrp* loci in *X. c.* pv. *vesicatoria* was recently identified. This "PIP" (plant-inducible promoter) box has the sequence TTCGC-N15-TTCGC and occurs upstream of the –35 consensus sequence in four out of six *hrp* promoters (S. Fenselau and U. Bonas, unpublished). Experiments are underway to test whether this is a protein binding motif.

6.4 *Xanthomonas campestris*

Expression of *hrp* genes in *X. c.* pv. *campestris* was determined after growth in vitro and found to be induced in a minimal medium with sucrose and/or fructose as carbon source. No expression occurred in complex media or with high concentrations of organic nitrogen (Arlat et al. 1991). In *X. c.* pv. *vesicatoria*, expression of the six *hrp* loci is induced in the plant but cannot be efficiently induced in the synthetic media tested so far. However, culture filtrates of sterile tomato cell suspension cultures (called TCM) induced expression of the six *hrp* loci in *X. c.* pv. *vesicatoria* whereas the basal Murashige-Skoog culture medium did not. The inducing factor(s) could only partially be purified from TCM and was found to be smaller than 1000 dalton, heat-stable, organic, and hydrophilic (Schulte and Bonas 1992a). De novo transcription of all *hrp* loci occurs rapidly within 1 h after transfer of the bacteria into TCM (S. Fenselau and U. Bonas, unpublished). A minimal medium was designed which would not suppress *hrp* gene induction. This medium, called XVM1, induces the *hrpF* locus (Fig. 1A) to high levels and differs from the other media described above, in particular by its low concentration in phosphate. Both sucrose and methionine are needed for efficient induction. It is not known whether a plant factor is necessary for activation of the other *hrp* loci, or if the XVM1 medium still lacks components or contains them in suppressing amounts (Schulte and Bonas 1992b).

6.5 *Erwinia* and *Pseudomonas solanacearum*

The *hrp* genes of *Erwinia amylovora* are rapidly induced in the non-host, tobacco, and more slowly in the host, pear. Several loci were induced in minimal medium with mannitol as a carbon source. Induction was suppressed by high concentrations of nitrogen and by glucose and was slightly temperature dependent (Wei et al. 1992b).

In *P. solanacearum*, the *hrp* cluster was also induced in host and in non-host plants, as well as in minimal medium. The best carbon sources for induction of four of the six transcription units were pyruvate and glutamate while, as in other bacteria, casamino acids suppressed induction (Arlat et al. 1992). The two rightmost *hrp* transcription units (5 and 6; Fig. 1B) are constitutively expressed but can be induced under certain conditions (Genin et al. 1992).

The only other gene reported to regulate *hrp* gene expression is *hrpB* from *P. solanacearum*. The gene is part of the *hrp* cluster and appears to be a member of the AraC family of positive regulatory proteins. Interestingly, *hrpB* is related to *virF* of *Yersinia* (Cornelis et al. 1989; Genin et al. 1992). The *hrpB* gene positively regulates four of the six *hrp* loci, as well as the *popA* locus, located outside of the *hrp* cluster which encodes a protein secreted in a Hrp-dependent way (see above; Arlat et al. 1994). Whether the HrpB protein binds directly to *hrp* promoters is not yet known.

At this time one can only speculate whether the regulatory systems for *hrp* gene expression employed by *P. solanacearum* and *P. syringae* are really different

or whether there is a global regulatory network thus allowing the fine tuning of gene expression in response to environmental cues. In conclusion, most *hrp* loci from different bacteria are inducible in a particular minimal medium. At this time it cannot be ruled out that stimulation of *hrp* gene expression involves specific plant factors as was described for the virulence genes of *Agrobacterium* (Winans 1992). Since the composition of the nutrients available to the pathogen in the plant is not known one can only speculate that the conditions described above reflect the situation in the plant. It is noteworthy that the in vitro culture will only mimic the dynamic nutritional situation that bacteria experience in their interaction with a plant for a short time. In mammalian bacterial pathogens, the expression of genes involved in virulence is also regulated in response to environmental cues rather than to specific host molecules. This subject has been reviewed recently (Mekalanos 1992 and in accompanying chapters), and I will only mention some important factors. In *Yersinia*, the *vir* and *lcr* genes are regulated by low calcium (low calcium response genes; Straley et al. 1993) and by temperature (Cornelis et al. 1989; see chapter by Cornelis). A calcium effect has not been described for any plant bacterium. In our laboratory no effect of calcium on *hrpF* gene expression in XVM1 was observed (Schulte and U. Bonas, unpublished). Expression of *invA* of *S. typhimurium* of the *mxi* and *ipa* genes of *Shigella* is affected by osmolarity and the later genes also by temperature (Galán and Curtiss 1990; Hale 1991).

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The Enigmatic Avirulence Genes of Phytopathogenic Bacteria

J.L. Dangl

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1 Action at the "Pathogenic Cusp"

The previous chapters have discussed how phytopathogenic bacteria can sense and respond to conditions present in a variety of microenvironments: soil, water, plant cell surfaces, and intracellular spaces. The switch from epiphyte to pathogen is apparently accompanied by fundamental reprogramming of gene activity and attendant function, as evidenced by induction of *hrp* genes and subsequent production of various virulence and pathogenicity factors, some of which are host-specific, some not. This reprogramming switch between epiphytic and pathogenic growth strategies, "the pathogenic cusp" (Dangl 1994), is the point at which not only the potential pathogen but also the host first sense and respond to each other. A successful plant defense response should be based on surveillance and interdiction before the pathogen has a chance to establish production of the armory of factors which determine successful colonization of that host. It is incumbent on each potential plant host, then, to evolve mechanisms to recognize some factor, preferably one produced at this pathogenic cusp, and to base resistance strategies on early recognition. Thus, an evolutionary tug-of-war is

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